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A Cell Type-specific Constitutive Point Mutant of the Common β-Subunit of the Human Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF), Interleukin (IL)-3, and IL-5 Receptors Requires the GM-CSF Receptor α-Subunit for Activation*

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The high affinity receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF) consists of a cytokine-specific α-subunit (hGMRα) and a common signal-transducing β-subunit (hβc) that is shared with the interleukin-3 and -5 receptors. We have previously identified a constitutively active extracellular point mutant of hβc, I374N, that can confer factor independence on murine FDC-P1 cells but not BAF-B03 or CTLL-2 cells (Jenkins, B. J., D'Andrea, R. J., and Gonda, T. J. (1995) *EMBO J.* 14, 4276–4287). This restricted activity suggested the involvement of cell type-specific signaling molecules in the activation of this mutant. We report here that one such molecule is the mouse GMRα (mGMRα) subunit, since introduction of mGMRα, but not hGMRα, into BAF-B03 or CTLL-2 cells expressing the I374N mutant conferred factor independence. Experiments utilizing mouse/human chimeric GMRα subunits indicated that the species specificity lies in the extracellular domain of GMRα. Importantly, the requirement for mGMRα correlated with the ability of I374N (but not wild-type hβc) to constitutively associate with mGMRα. Expression of I374N in human factor-dependent UT7 cells also led to factor-independent proliferation, with concomitant up-regulation of hGMRα surface expression. Taken together, these findings suggest a critical role for association with GMRα in the constitutive activity of I374N.

GM-CSF[1] is a potent cytokine that promotes the survival, proliferation, differentiation, and functional activity of a wide variety of hemopoietic cell types including monocytes/macrophages, granulocytes, and myeloid progenitor cells (reviewed in Ref. 1). Like other cytokines, GM-CSF exerts its biological activities through binding to specific receptors on the surface of target cells. The high affinity receptor for human GM-CSF (hGMR) is composed of a cytokine-specific α-subunit (hGMRα) associated with a common signal-transducing β-subunit (hβc) that is also utilized by the IL-3 and IL-5 receptors (2–6), all of which belong to the cytokine receptor family (reviewed in Ref. 7). Members of this family are characterized by a structurally conserved extracellular cytokine receptor module (CRM) of about 200 amino acids that consists of two fibronectin type III-like domains (8). The β-subunit has two CRMs, whereas the α-subunits contain one CRM and an additional N-terminal domain of about 100 amino acids.

Although the stoichiometry of subunits in active hGMR, hIL-3R, and hIL-5R complexes remains unresolved, it has become clear that ligand-induced α-β-subunit heterodimerization is a key step in the formation of these complexes (9, 10). More recently, it has been shown that β-subunit homodimers are found in active hGMR (11) and human IL-3R (12) complexes and that the functional hGMR complex may contain at least two α-subunits (13). Taken together, these results suggest that the α- and β-subunits may form higher order receptor complexes, and indeed it has been proposed that the GMR/IL-3R/IL-5R normally functions as an $\alpha_2\beta_2$ tetramer (10, 12, 13).

The isolation of constitutively active cytokine receptor mutants has provided a useful tool for examining the normal activation process of some receptors (*e.g.* erythropoietin receptor and c-Mpl (14, 15)), since these mutant receptors most likely mimic the structure of the normal cytokine-activated receptors. With regard to the GMR/IL-3R/IL-5R system, we have previously combined random mutagenesis with retroviral expression cloning to identify constitutively activating point mutations in hβc by virtue of their ability to confer factor-independent proliferation on mouse factor-dependent FDC-P1 cells (16, 17). One of these mutations, V449E, is located in the transmembrane domain of hβc and is similar to an activating mutation in the *neu*/c-*erb*B-2 oncogene (18, 19). By analogy, this mutant most likely acts by inducing hβc homodimerization. Another group of activating point mutations, exemplified by I374N, lies in the extracellular region of hβc; however, it is unclear precisely how this group might affect receptor function. Interestingly, only certain transmembrane mutants, such as V449E, were able to confer factor independence on mouse factor-dependent BAF-B03 cells, suggesting that the I374N mutation activates hβc in a cell type-specific manner.

One possible explanation for the cell type specificity of the I374N mutant is that a molecule that is present in FDC-P1

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[1] The abbreviations used are: GM-CSF granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; GMR, GM-CSF receptor; hGMR, human GMR; mGMR, mouse GMR; GMRα, GMR α-subunit; IL, interleukin; hIL, human IL; IL-3R and IL-5R, interleukin-3 and -5 receptors, respectively; βc, common β-subunit of the GM-CSF, IL-3 and IL-5 receptors; hβc, human βc; CRM, cytokine receptor module; PCR, polymerase chain reaction; kb, kilobase pair(s); wt, wild type; HSV, herpes simplex virus.

(and other myeloid) cells is required for its constitutive activity. We report here the use of retroviral expression cloning to identify the mouse GMRα (mGMRα) subunit as one such molecule and show that one effect of the I374N mutation is to induce constitutive association with mGMRα.

EXPERIMENTAL PROCEDURES

Cell Lines—BOSC 23 (20) and Ψ2 (21) ecotropic retroviral packaging cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The BING amphotropic retroviral packaging cell line was kindly provided by Prof. Suzanne Cory (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) with permission from Dr. Warren Pear (MIT, Cambridge, MA) and was maintained as described above. The CTL-EN subline of the mouse IL-2-dependent cell line, CTLL-2 (22), was kindly provided by Dr. John Norton (Paterson Institute for Cancer Research, Manchester) and was maintained as described previously for CTLL-2 cells (16). Mouse IL-3-dependent BAF-B03 cells (23) were maintained as described previously (16). Human factor-dependent UT7 cells (24) were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum supplemented with 2 ng/ml human GM-CSF.

Construction of the FDC-P1 cDNA Library—cDNA library construction was performed essentially as described by Rayner and Gonda (25). Briefly, cDNA was synthesized from the mouse IL-3/GM-CSF-dependent myeloid cell line FDC-P1 (26) and size-selected for cDNA fragments greater than 500 base pairs. Following digestion with *Bam*HI and *Xho*I, the size-selected cDNA was ligated directionally into the pRUFNeo retroviral expression vector (25). The library was amplified in *Escherichia coli* by electroporation of aliquots of the ligated FDC-P1 cDNA. The resultant colonies from each electroporation were harvested, and plasmid DNA was prepared from each pool.

Infection of Target Cells with the FDC-P1 cDNA Library—Retroviral DNA was used to generate a library of retroviruses by a modification of the method described by Rayner and Gonda (25). Briefly, amphotropic BING packaging cells were transiently transfected using the procedure described by Jenkins *et al*. (27) with 10 μg of retroviral plasmid per 60-mm culture dish (seeded 18 h previously with 2×10^6 cells). At 48 h post-transfection, virus-containing supernatants were filtered and used to infect ecotropic Ψ2 packaging cells. Infected Ψ2 cells were harvested and selected in medium containing G418 (400 μg/ml) to generate the stable G418-resistant Ψ2 retroviral library. BAF-B03 cells expressing the I374N hβc mutant were infected with the Ψ2 retroviral library by co-cultivating 3.75×10^5 BAF/I374N cells with 1.2×10^6 irradiated (30 grays) Ψ2 cells for 48 h in each of eight 25-cm^2 culture flasks. The BAF/I374N cells were then harvested, washed, and selected for factor-independent growth in 24-well multidishes (204 wells, each seeded with 10^5 cells) in liquid culture medium without factor.

PCR Recovery and Sequencing of cDNAs from Factor-independent Cells—PCR was performed on 100 ng of genomic DNA (prepared essentially as described by Hughes *et al*. (28)) with an XL PCR kit (Perkin-Elmer) under conditions recommended by the manufacturer. The primers used for amplification were RCF1 (25), which corresponds to the vector *gag* sequence approximately 80 base pairs 5′ of the polylinker in the pRUFNeo vector and RCR2 (5′-ATAGCCTCTCCACCCAAGCG-3′), which corresponds to the MC1*Neo* sequence 364 base pairs 3′ of the polylinker. PCR products were agarose gel-purified, and the 5′- and 3′-ends were sequenced with PCR primers. Internal primers corresponding to cDNA sequences obtained from initial sequencing with PCR primers were subsequently used to fully sequence PCR products. Sequencing reactions were performed using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer), and sequence data were obtained by running reactions on an ABI Prism 377 DNA Sequencer.

Receptor Expression Constructs—The pRUFNeo/mGMRα expression construct was generated by subcloning the full-length mGMRα cDNA recovered from factor-independent BAF/I374N infectants into the *Bam*HI and *Hin*dIII restriction sites of pRUFNeo. The pRUFNeo/hGMRα expression construct was generated by inserting the cDNA for hGMRα into the *Xho*I site of pRUFNeo.

To introduce the 8-amino acid DYKDDDDK FLAG polypeptide (Eastman Kodak Co.) at the N terminus of mGMRα (^{F}mGMRα), a 5′ *Bam*HI/*Nae*I fragment encoding the signal sequence and first 8 structural residues of mGMRα was excised from pRUFNeo/mGMRα and replaced in frame with a PCR-generated *Bam*HI/*Nae*I fragment from pcDNA1Neo/^{F}hIL-3Rα (kindly provided by Richard D'Andrea, Hanson Center for Cancer Research, Adelaide, South Australia, Australia) encoding the hIL-3Rα signal sequence, FLAG octapeptide, and first 6 structural residues of hIL-3Rα. The sense primer corresponded to the T7 promoter sequence and included a *Bam*HI site, and the antisense primer corresponded to codons 19–24 (as numbered by Kitamura *et al*. (5)) of hIL-3Rα and included a *Nae*I site. The pRUFPuro/^{F}mGMRα expression vector was constructed by inserting the *Bam*HI/*Eco*RI ^{F}mGMRα cDNA from pRUFNeo/^{F}mGMRα into the *Bam*HI and *Eco*RI sites of the pRUFPuro retroviral expression vector (16).

The HSV-derived 11-amino acid QPELAPEDPED polypeptide (Novagen) was inserted after the signal sequence of the wild-type and I374N mutant β-subunits (between residues Cys[16] and Trp[17] as numbered by Hayashida *et al*. (4)) by site-directed mutagenesis using the pAlter-1 system (Promega) in accordance with the manufacturer's instructions. The modified β-subunit cDNAs were subcloned into the *Bam*HI and *Hin*dIII restriction sites of pRUFNeo.

The following GMRα chimeras were generated by PCR amplification and ligation of the relevant portions of human and mouse GMRα: (i) the pRUFNeo/hαmα1 chimera encoding the extracellular and transmembrane domains of hGMRα (346 amino acids) and the cytoplasmic domain of mGMRα (38 amino acids); (ii) the pRUFNeo/hαmα2 chimera encoding the extracellular N-terminal domain of hGMRα (117 amino acids) and the extracellular CRM, transmembrane, and cytoplasmic domains of mGMRα (262 amino acids); (iii) the pRUFNeo/^{F}mαhα1 chimera encoding the extracellular and transmembrane domains of ^{F}mGMRα (335 amino acids) and the cytoplasmic domain of hGMRα (54 amino acids); and (iv) the pRUFNeo/^{F}mαhα2 chimera encoding the extracellular FLAG-tagged N-terminal domain of ^{F}mGMRα (111 amino acids) and the extracellular CRM, transmembrane, and cytoplasmic domains of hGMRα (283 amino acids). A full description of the templates and primers used is available upon request.

Extracellular truncations of mGMRα were generated by PCR on the pRUFNeo/^{F}mGMRα construct with primers designed to amplify the entire construct except for the desired extracellular sequence to be removed while leaving the N-terminal signal sequence and FLAG octapeptide intact. Each PCR was performed with different sense primers corresponding to codons 97–102 (for mαD1) and codons 195–200 (for mαD2) and the same antisense primer corresponding to codons 9–14 of mGMRα. The blunt ends of each PCR fragment were then ligated together in frame.

The cytoplasmic truncation mutant of mGMRα was generated by PCR on the pRUFNeo/^{F}mGMRα construct with RCF1 as the sense primer and an antisense primer that contained codons 344–339 of the mGMRα cytoplasmic domain together with a *Hin*dIII restriction site and termination codon. The PCR products were subcloned into the *Bam*HI and *Hin*dIII restriction sites of pRUFNeo.

All PCRs were performed on 20 ng of plasmid DNA with *Pfu* DNA polymerase (Stratagene) under conditions recommended by the manufacturer. The structures of all mutated or chimeric cDNAs were verified by sequencing.

Infection of Hemopoietic Cells—Retroviral infection of mouse BAF-B03 cells and CTL-EN cells was performed using either stably transfected Ψ2 packaging cells (16) or transiently transfected BOSC 23 packaging cells as described previously (27). Infected BAF-B03 cells were selected in liquid culture medium containing growth factor and either G418 (1.5 mg/ml) or puromycin (2 μg/ml). Infected CTL-EN cells were selected as described previously for CTLL-2 cells (16).

Retroviral infection of human UT7 cells was performed using amphotropic BING packaging cells based on the method for infecting mouse hemopoietic cells with BOSC 23-derived retroviruses (27). Briefly, BING cells were transiently transfected with 10 μg of retroviral DNA, following which infections were performed by co-cultivating 3×10^5 UT7 cells with the BING cells for 48 h in growth medium supplemented with 4 μg/ml polybrene. Cells were harvested and selected in liquid culture medium containing growth factor and G418 at 1.5 mg/ml.

Analysis of Receptor Subunit Expression by Flow Cytometry—Expression of receptor subunits on the surface of infected cells was detected by high sensitivity immunofluorescence followed by flow cytometry on an Epics-Profile II analyzer (Coulter). High sensitivity immunofluorescence was performed by incubating cells with primary antibody followed by biotinylated anti-mouse IgG (Vector Laboratories) and streptavidin-phycoerythrin (Caltag Laboratories). Expression of FLAG epitope-tagged mGMRα subunits was detected by staining with the anti-FLAG monoclonal antibody M2 (Kodak), and expression of hGMRα subunits was detected by staining with the anti-hGMRα monoclonal antibody 8G6 (29). Expression of wild-type and I374N mutant β-subunits on the surface of infected BAF-B03 cells was detected by staining with the anti-hβc monoclonal antibody 1C1 (10), whereas HSV epitope-tagged wild-type and I374N mutant β-subunits expressed on the surface of human UT7 cells were detected by staining with an HSV tag monoclonal antibody (Novagen).

Cell Proliferation Assays—Infected cells were washed twice, and triplicate samples of equal cell number (5×10^3) were cultured in a 96-well microtiter plate with or without appropriate growth factor for 72 h. Cell proliferation was measured by the CellTiter 96 nonradioactive cell proliferation assay (Promega).

Immunoprecipitation and Immunoblotting—Cells (2×10^7) were cultured overnight in the absence of growth factor and left unstimulated. Cells were washed with cold PBS containing 20 mM sodium orthovanadate and lysed on ice in lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 2 mg/ml iodoacetamide, 0.2 mg/ml trypsin inhibitor (Boehringer Mannheim), and Complete™ protease inhibitor (Boehringer Mannheim)) for 15 min. Insoluble material was removed by centrifugation, and cell lysates were incubated with primary antibody for 2 h at 4 °C. Antibodies used for immunoprecipitation were the anti-hβc antibody 8E4 (30) and the anti-FLAG antibody M2 (Kodak). Immune complexes were precipitated with 75 μl of protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C, washed three times with lysis buffer, and boiled in 1× reducing SDS sample buffer. In the case of whole cell protein analyses, samples were lysed in buffer without 10% glycerol, and insoluble material was removed and boiled in 1× reducing SDS sample buffer.

Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and electrophoretically transferred to Poly-Screen® polyvinylidene difluoride membranes (NEN Life Science Products). Membranes were then incubated with the anti-hβc antibody 1C1 (10), the anti-hGMRα antibody 8D10 (29), or the biotinylated anti-FLAG antibody BIOM2 (Kodak), as indicated, following which the membranes were washed and incubated with either an alkaline phosphatase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) or a streptavidin-conjugated alkaline phosphatase antibody (Molecular Probes, Inc., Eugene, OR), as appropriate. Membranes were washed and subjected to enhanced chemifluorescence detection (Amersham Pharmacia Biotech) as per the manufacturer's instructions, following which they were scanned on a FluorImager (Molecular Dynamics, Inc., Sunnyvale, CA). For reprobing, membranes were stripped in 50 mM Tris (pH 7.4), 2% SDS, 100 mM β-mercaptoethanol at 55 °C for 20 min; washed; and subsequently probed with the indicated antibodies.

RESULTS

Isolation of Factor-independent BAF/I374N Cells Infected with an FDC-P1 cDNA Retroviral Expression Library—We have previously identified a constitutively activating point mutation, I374N, in the extracellular region of hβc by virtue of its ability to confer factor-independent growth on FDC-P1 cells (16). Surprisingly, this mutant was unable to confer factor independence on mouse IL-3-dependent BAF-B03 cells, leading us to suggest that the cell type-specific activity of this mutant may reflect the presence of a β-subunit-associated signaling molecule in FDC-P1 cells, but not in BAF-B03 cells, that is required by this mutant for constitutive activation (16). We therefore reasoned that the introduction of such a molecule from FDC-P1 cells into BAF-B03 cells expressing the I374N mutant should lead to its constitutive activity and thus render these cells factor-independent.

Using procedures described previously (25), an FDC-P1 cDNA library (~8.5×10^5 independent plasmid clones, with an average insert size of 1.1 kb) was generated in the pRUFNeo retroviral expression vector. As described under "Experimental Procedures," the plasmid DNA was used to generate a stable Ψ2 retroviral library estimated to contain ~3.5×10^6 independent viral producer clones, which should adequately represent all cDNA species present in the plasmid library.

BAF-B03 cells expressing I374N (BAF/I374N) were infected by co-cultivation with the virus-producing Ψ2 cells at an infection frequency of 18% (estimated by colony assays in the presence of G418). As a control, parallel infections were also performed on uninfected BAF-B03 cells and BAF-B03 cells expressing wild-type hβc. Cells were then selected for factor-independent growth in 24-well multidishes. After 1 week in the absence of factor, 37 of 204 wells seeded with 10^5 infected BAF/I374N cells contained viable, proliferating cells, while no

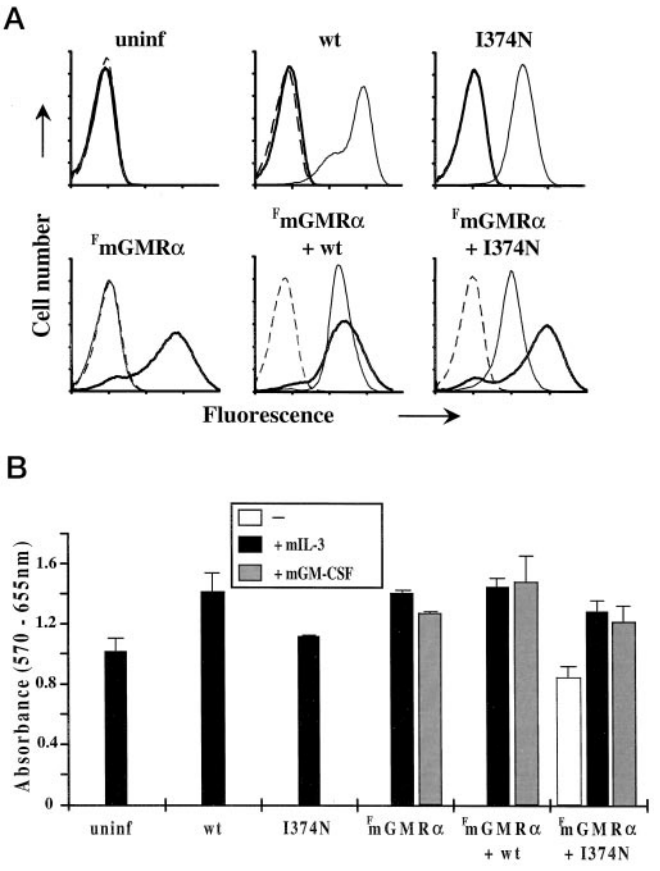

FIG. 1. **Co-expression of ^{F}mGMRα with I374N confers factor independence on BAF-B03 cells.** *A*, flow cytometric analysis of ^{F}mGMRα and β-subunit expression on G418-selected BAF-B03 cells. Uninfected BAF-B03 cells (*uninf*) or cells expressing either wild-type (*wt*) or I374N β-subunits were infected with a retrovirus encoding the ^{F}mGMRα subunit and stained with an irrelevant control antibody (*dashed line*), the anti-hβc antibody 1C1 (*thin solid line*), and the anti-FLAG antibody M2 (*thick solid line*) by high sensitivity immunofluorescence. Cell number and fluorescence are in arbitrary units; the latter is plotted on a logarithmic scale. Also shown are analyses of cells not exposed to the ^{F}mGMRα virus. *B*, proliferation of the BAF-B03 cells depicted in *A* in the presence of mIL-3 (300 units/ml) or mGM-CSF (80 units/ml) or in the absence of either factor, as indicated. Proliferation assays were carried out, as described under "Experimental Procedures," with 5×10^3 cells plated in triplicate. *Error bars* indicate the S.E. of the mean of each triplicate.

such cells were present in control cultures. Factor independence was not the result of autocrine growth factor production, since conditioned medium from the factor-independent cell cultures did not support the growth of uninfected BAF-B03 cells (data not shown).

PCR Recovery of Mouse GMRα cDNA from Factor-independent BAF/I374N Infectants—To identify the cDNA sequence carried by the provirus in the factor-independent BAF/I374N infectants, long range PCR was performed with retroviral primers on genomic DNA samples from 17 of the 37 factor-independent cell populations. This revealed a common fragment of approximately 2.3 kb that was amplified from all 17 genomic DNA samples (data not shown); considering the positions of the PCR primers relative to the cloning sites in pRUFNeo, the size of the cDNA insert was estimated to be 1.9 kb. For 8 of the 17 samples, the 2.3-kb fragment was the only PCR product generated, suggesting that these factor-independent cell populations contained only one retroviral insertion and that its presence was responsible for factor independence. Sequence analysis of the 1.9-kb cDNA insert recovered from two of the factor-independent cell populations revealed that it cor-

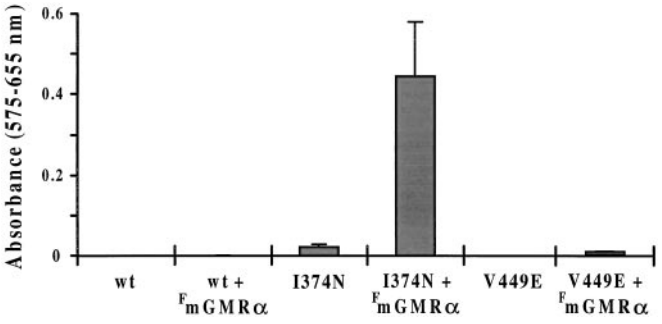

FIG. 2. **Factor-independent proliferation of CTL-EN cells co-expressing ^{F}mGMRα and the I374N mutant.** Proliferation of CTL-EN cells expressing the indicated subunits in the absence of factor. Cells were maintained in mouse IL-2 (4 ng/ml) and the appropriate drug selection prior to assay for factor-independent growth.

responded to the full-length cDNA for the mGMRα subunit (31).

Expression of mGMRα with I374N in BAF-B03 and CTL-EN Cells Results in Factor Independence—To confirm that mGMRα would allow the constitutive activation of I374N, we expressed the recovered mGMRα subunit in BAF/I374N cells and then tested these cells for factor independence. In order to monitor cell surface expression of mGMRα, a FLAG epitope-tagged mGMRα (^{F}mGMRα) was generated in the pRUFNeo vector (see "Experimental Procedures"). This was introduced into puromycin-resistant BAF/I374N cells as well as wild-type hβc-expressing and uninfected BAF-B03 cells. Following selection for G418 resistance, flow cytometric analysis with a FLAG-specific monoclonal antibody indicated that the ^{F}mGMRα subunit was efficiently expressed on the surface of these cells (Fig. 1*A*). Upon selection for growth in medium without factor, only BAF-B03 cells co-expressing ^{F}mGMRα and I374N exhibited factor-independent growth (Fig. 1*B*). The ability of ^{F}mGMRα to behave as wild-type mGMRα was demonstrated by the proliferation of all ^{F}mGMRα-infected BAF-B03 cells in response to mGM-CSF (Fig. 1*B*).

The observation that the mouse GMRα subunit was required for the activity of I374N raised the possibility that another component(s) of the mouse GMR or IL-3R (*i.e.* mIL-3Rα mβc or mβIL-3) present in FDC-P1 and BAF-B03 cells might also be needed. We therefore introduced I374N and, as a control, wild-type hβc with ^{F}mGMRα into mouse IL-2-dependent CTL-EN cells, which do not express any receptor components belonging to the GMR or IL-3R. CTL-EN cells are a derivative of CTLL-2 cells engineered for increased expression of the ecotropic retroviral receptor (41),[2] thereby rendering them more susceptible to retroviral infection. We also included the V449E transmembrane hβc mutant in this experiment, since it is inactive when expressed in CTLL-2 cells, although, unlike the I374N mutant, it does confer factor independence on BAF-B03 cells (16). The expression of these subunits was confirmed by flow cytometry (data not shown), following which these cells were tested for factor-independent proliferation. As shown in Fig. 2, only CTL-EN cells expressing both ^{F}mGMRα and I374N were factor-independent, thereby indicating that components of the mouse IL-3R are not required for the constitutive activity of I374N. In view of this result, all subsequent experiments were performed in BAF-B03 cells.

The I374N Mutation Induces Constitutive Association of hβc with mGMRα in BAF-B03 Cells—To examine whether the requirement for mGMRα by I374N might reflect a physical association between these two subunits, BAF-B03 cells co-expressing ^{F}mGMRα with I374N or, as a control, wild-type hβc were

[2] J. Norton, personal communication.

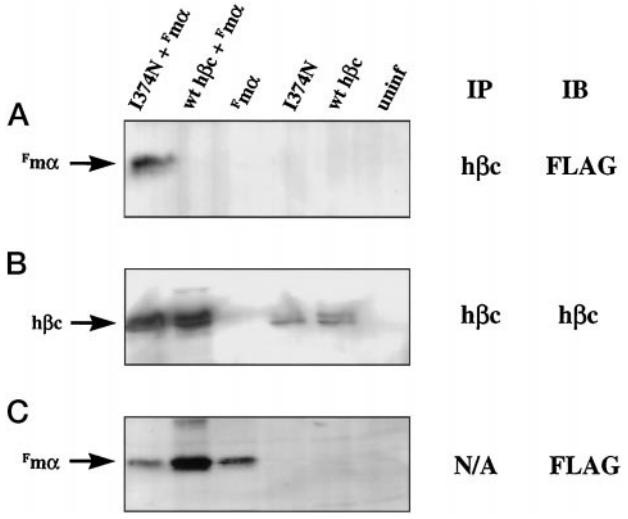

FIG. 3. **Co-immunoprecipitation of ^{F}mGMRα and I374N from BAF-B03 cells.** *A* and *B*, uninfected BAF-B03 cells and cells expressing the indicated subunits were incubated in medium without factor, and lysates were immunoprecipitated (*IP*) with the anti-hβc antibody 8E4. Immunoprecipitated proteins were analyzed by immunoblotting (*IB*) with the anti-FLAG antibody M2 (*A*) or with the anti-hβc antibody 1C1 (*B*). *C*, whole cell lysates from the indicated BAF-B03 cells were subjected to immunoblotting with the anti-FLAG antibody M2.

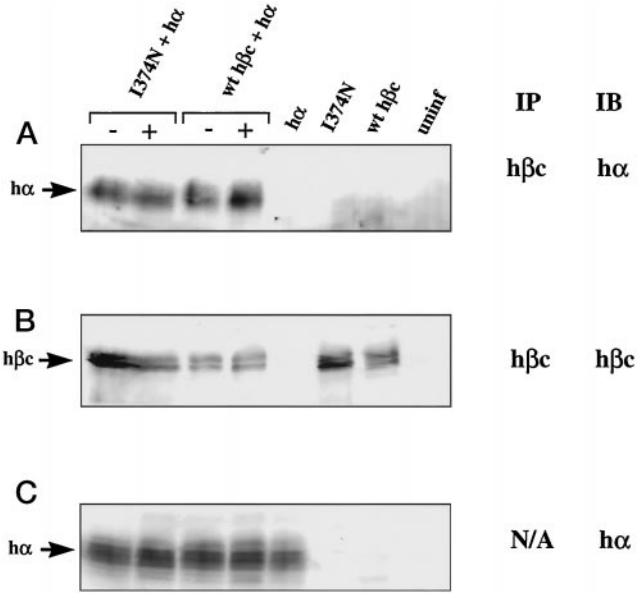

FIG. 4. **Ligand-independent association of hGMRα with I374N and wild-type hβc.** *A* and *B*, uninfected BAF-B03 cells and cells expressing the indicated hGMR subunits were incubated in medium with (+) or without (−) hGM-CSF (10 ng/ml), and lysates were immunoprecipitated (*IP*) with the anti-hβc antibody 8E4. Immunoprecipitated proteins were analyzed by immunoblotting (*IB*) with the anti-hGMRα antibody 8D10 (*A*) or with the anti-hβc antibody 1C1 (*B*). *C*, whole cell lysates from the indicated BAF-B03 cells were subjected to immunoblotting with the anti-hGMRα antibody 8D10.

subjected to immunoprecipitation with an anti-hβc antibody, followed by immunoblot analysis with an anti-FLAG antibody. As shown in Fig. 3*A*, a protein of 60-kDa, consistent with the predicted size of mGMRα, was detected only in immunoprecipitates from cell lysates expressing ^{F}mGMRα and the I374N mutant. Importantly, the converse immunoprecipitation (with anti-FLAG antibody) and immunoblot analysis (with anti-hβc antibody) confirmed the physical association between mGMRα and the I374N mutant (data not shown). Reprobing the immunoblot with an anti-hβc antibody indicated that both wild-type

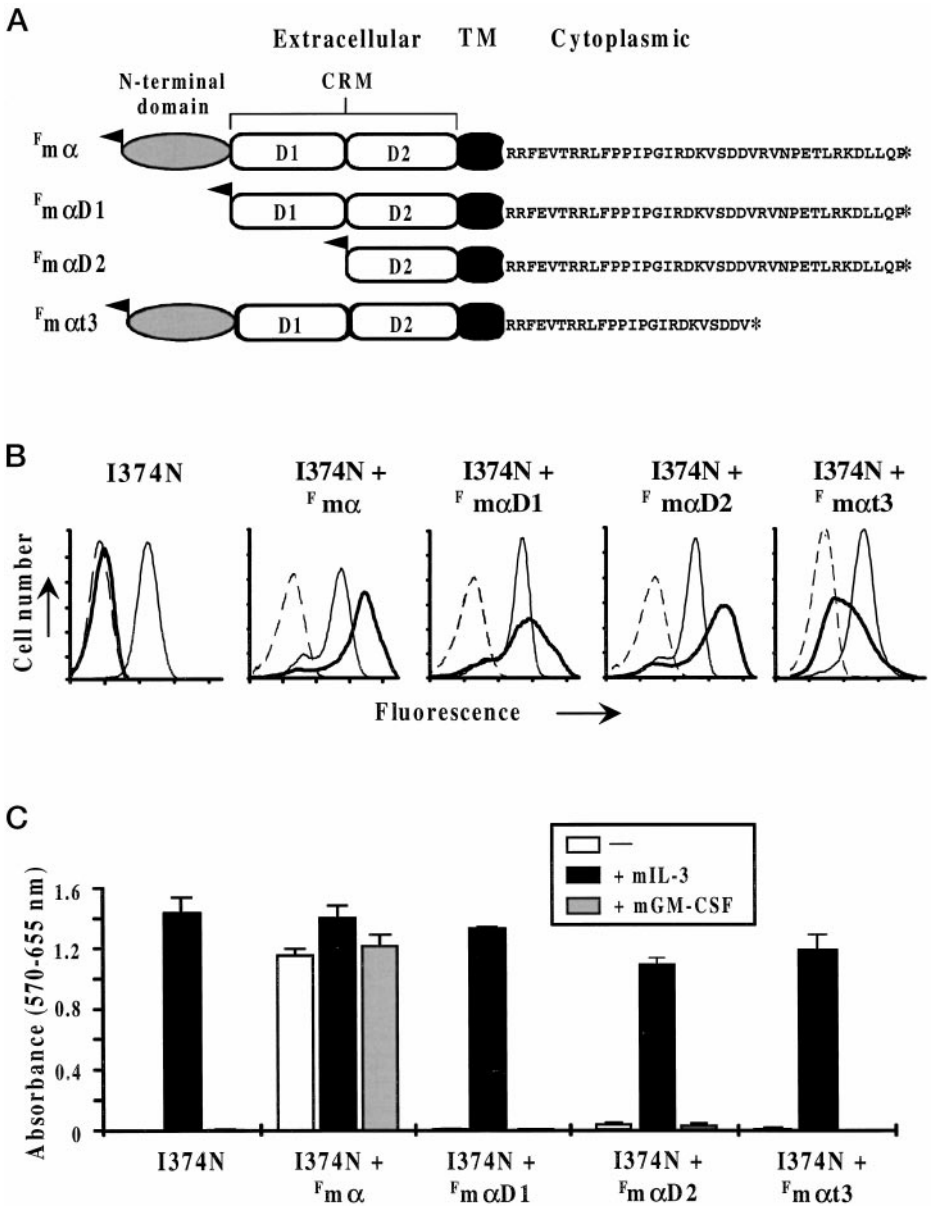

FIG. 5. **Analysis of BAF/I374N cells expressing ^{F}mGMRα extracellular and cytoplasmic truncation mutants.** *A*, schematic illustration of the truncated ^{F}mGMRα subunits showing the regions deleted in each truncation mutant. For comparison, the full-length ^{F}mGMRα is also shown. The *asterisks* represent the C terminus of the depicted subunits. *B*, flow cytometric analysis of BAF/I374N cells superinfected with full-length and truncated ^{F}mGMRα subunits. Procedures, nomenclature, and axes are as in Fig. 1*A*. Also shown are "parental" BAF/I374N cells. *C*, proliferation assay of the BAF/I374N cells depicted in *B* in the presence of mIL-3 (300 units/ml) or mGM-CSF (80 units/ml) or in the absence of either factor, as indicated.

and I374N β-subunits were immunoprecipitated from the appropriate cell lysates (Fig. 3*B*). Furthermore, immunoblot analysis of whole cell lysates with an anti-FLAG antibody indicated that the total levels of ^{F}mGMRα protein present in lysates from all cell populations were comparable (Fig. 3*C*). Together, these observations indicate that the I374N mutation acts, at least in part, by inducing constitutive association of hβc with mGMRα.

The constitutive association of mGMRα with the I374N mutant was reminiscent of the ability of human GMRα to associate with wild-type hβc in the absence of GM-CSF (32). We therefore examined the ability of I374N to associate with hGMRα in the absence of ligand, since a failure to do so could explain our previous observation that co-expression of hGMRα did not allow constitutive activity of I374N in BAF-B03 cells (Refs. 16 and 27; Fig. 7). The experiment illustrated in Fig. 4*A* shows, however, that both mutant and wild-type hβc could associate equally well with hGMRα in the absence (or presence) of ligand, as judged by co-immunoprecipitation from BAF-B03 cells expressing both subunits. Equivalent levels of expression of the β- and α-subunits are confirmed by the analyses of Fig. 4, *B* and *C*, respectively.

Both the N-terminal and C-terminal Regions of mGMRα Are Essential for Activation of and Association with the I374N Mutant—To broadly define the regions of the mGMRα extracellular domain required for the constitutive activation of I374N, two FLAG-tagged extracellular truncation mutants were generated. One of these, ^{F}mαD1, lacked residues Leu^{15}-Ala^{96}, which comprise the N-terminal domain, whereas the other, ^{F}mαD2, lacked residues Leu^{15}-Glu^{194}, which also includes domain 1 of the cytokine receptor module (CRM; Fig. 4*A*). Although these truncation mutants (and full-length ^{F}mGMRα) were efficiently expressed on the surface of G418-resistant cells (Fig. 4*B*), neither truncation mutant was able to confer factor independence on BAF/I374N cells (Fig. 4*C*), indicating that the N-terminal domain of mGMRα is required for constitutive activation of I374N. Furthermore, the inability of BAF/I374N cells expressing the mαD1 mutant to proliferate in the presence of mGM-CSF suggests that the N-terminal domain of mGMRα is also important in normal mGMR function.

Considering that the cytoplasmic domain of GMRα is essential for normal GM-CSF-mediated cell growth (33), we also investigated whether the cytoplasmic domain of mGMRα was required for constitutive signaling by I374N. We therefore generated a cytoplasmic truncation mutant, ^{F}mαt3, which lacked

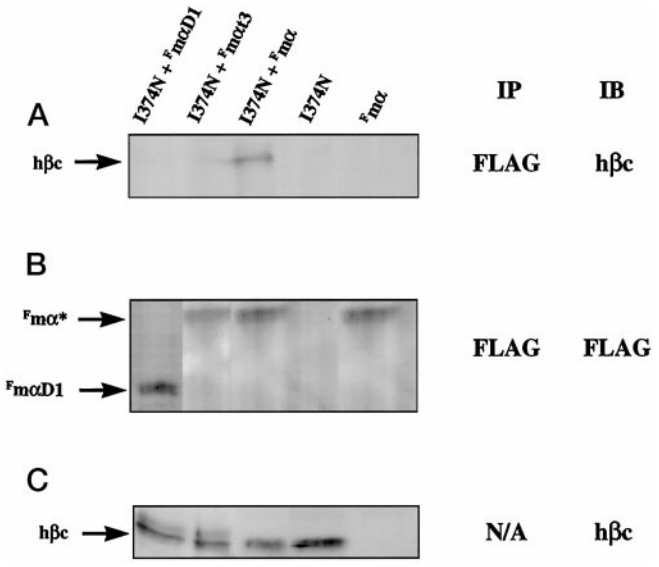

FIG. 6. **Extracellular and cytoplasmic truncations of ^{F}mGMRα abolish the co-immunoprecipitation of ^{F}mGMRα and I374N from BAF-B03 cells.** *A* and *B*, the BAF-B03 cells expressing the indicated subunits were incubated in medium without factor and lysates were immunoprecipitated (*IP*) with the anti-FLAG antibody M2. Immunoprecipitated proteins were analyzed by immunoblotting (*IB*) with the anti-hβc antibody 1C1 (*A*) or with the anti-FLAG antibody M2 (*B*). The *asterisk* by ^{F}mα in *B* indicates that the small C-terminal deletion mutant ^{F}mαt3 ran at the same size as the full-length ^{F}mGMRα under the gel conditions employed. *C*, whole cell lysates from the indicated BAF-B03 cells were subjected to immunoblotting with the anti-hβc antibody 1C1.

the C-terminal 14 amino acids of mGMRα (Fig. 5*A*). Although G418-resistant BAF/I374N infectants efficiently expressed ^{F}mαt3 (Fig. 5*B*), these cells failed to grow in the absence of factor (Fig. 5*C*) or in response to mGM-CSF. This implies that the C-terminal 14 amino acids of mGMRα are essential for mediating factor-independent growth conferred by I374N and also for normal mGM-CSF-mediated growth.

We next examined whether the inability of the extracellular and cytoplasmic truncation mGMRα mutants to confer factor independence on BAF/I374N cells was due to a failure to associate with I374N. Lysates from BAF/I374N cells expressing the ^{F}mαD1 extracellular truncation and the ^{F}mαt3 cytoplasmic truncation were therefore subjected to immunoprecipitation with an anti-FLAG antibody, followed by immunoblot analysis with an anti-hβc antibody. As shown in Fig. 6*A*, the I374N mutant was precipitated when co-expressed with the full-length ^{F}mGMRα subunit but not with the truncated ^{F}mGMRα subunits. Reprobing with an anti-FLAG antibody demonstrated that both full-length and truncated ^{F}mGMRα subunits were themselves immunoprecipitated (Fig. 6*B*), and immunoblot analysis of whole cell lysates with an anti-hβc antibody confirmed that comparable levels of the I374N mutant were expressed in the cells (Fig. 6*C*). Thus, these data demonstrate that both the N-terminal and C-terminal regions of mGMRα are essential for the association with I374N and, together with the data presented in Fig. 5, that the constitutive activity of I374N is dependent upon this association.

Species Specificity of GMRα for the Constitutive Activation of I374N Lies in Its Extracellular and/or Transmembrane Domains—In view of our previous observations that co-expression of the human GMRα subunit with I374N in BAF-B03 and CTLL-2 cells did not lead to factor-independent growth (16, 27), the ability of the mouse GMRα subunit to facilitate constitutive activity of I374N in BAF-B03 and CTL-EN cells was somewhat surprising. To define which region(s) of the GMRα subunit govern this apparent species specificity, we constructed a series of chimeric GMRα subunits containing regions from both species (Fig. 7*A*). These chimeras, along with the normal ^{F}mGMRα and hGMRα subunits, were then introduced into BAF/I374N cells and tested for their ability to confer factor independence. Flow cytometric analyses confirmed that while the chimeric GMRα subunits were co-expressed with the I374N mutant (Fig. 7*B*), only cells co-expressing the ^{F}mαhα1 chimera or, as expected, the normal ^{F}mGMRα subunit with the I374N mutant exhibited factor-independent proliferation (Fig. 7*C*). Thus, the species specificity lies in the extracellular and/or transmembrane domains of mGMRα. Furthermore, since chimeras containing only the mouse N-terminal domain (^{F}mαhα2) or the mouse extracellular CRM and transmembrane domain (hαmα2) were unable to confer factor independence on BAF-B03 cells, it is likely that both of the mGMRα regions present in these chimeras contribute to the species-specific requirement for mGMRα for I374N activity.

The I374N Mutant Confers Factor Independence on Human Hemopoietic Cells: A Possible Role for hGMRα in the Constitutive Activity of I374N in Human Cells—Although the human GMRα subunit was unable to facilitate the constitutive activity of I374N in mouse BAF-B03 and CTLL-2 cells (16, 27) (see also Fig. 7), it was conceivable that the I374N mutant might be constitutively active in human cells expressing hGMRα. We therefore introduced this mutant and, as a control, wild-type hβc into human GM-CSF/IL-3/erythropoietin-dependent UT7 cells and tested these cells for factor-independent proliferation. To distinguish between the introduced β-subunits and the endogenous β-subunits expressed by UT7 cells, we inserted an 11-amino acid HSV-derived epitope at the N terminus of both wild-type and I374N β-subunits. Cells infected with these modified β-subunits were then selected for G418 resistance or growth in medium without factor. The surface expression of the introduced subunits was confirmed by flow cytometric analysis of infected cells stained with both anti-hβc and anti-HSV antibodies (Fig. 8*A*). In two independent experiments, one of which is shown in Fig. 8*B*, the I374N mutant allowed factor-independent proliferation of UT7 cells. Factor independence was not the result of low level autocrine growth factor production, since conditioned medium from factor-independent cell pools did not support the growth of uninfected UT7 cells (data not shown).

Unfortunately, to the best of our knowledge, no human factor-dependent hemopoietic cell lines "equivalent" to BAF-B03 cells, *i.e.* that lack human GMRα, are available; thus, we could not directly test the requirement for human GMRα by I374N in human hemopoietic cells. Notably, however, flow cytometric analysis with an anti-hGMRα antibody revealed that the expression of hGMRα was significantly up-regulated on the surface of factor-independent cells expressing I374N (*FI *I374N cells*) compared with uninfected cells or G418-resistant cells (expressing wild-type hβc or I374N) that were not selected for factor independence (Fig. 8*C*). Importantly, the increase in hGMRα expression specifically correlated with the factor independence of I374N-expressing cells. This increase in hGMRα expression was not simply a function of high level β-subunit expression (see *FI *I374N histogram* in Fig. 8*A*), since infected UT7 cells that were sorted for comparably high levels of HSV-tagged wild-type hβc exhibited a similar low level of hGMRα expression to the unsorted cells (**wt*) shown in Fig. 8*C* (data not shown).

DISCUSSION

Constitutive Activation of I374N in Mouse Cells Requires mGMRα—The I374N mutation in the extracellular domain of hβc confers factor independence on mouse FDC-P1 cells but not

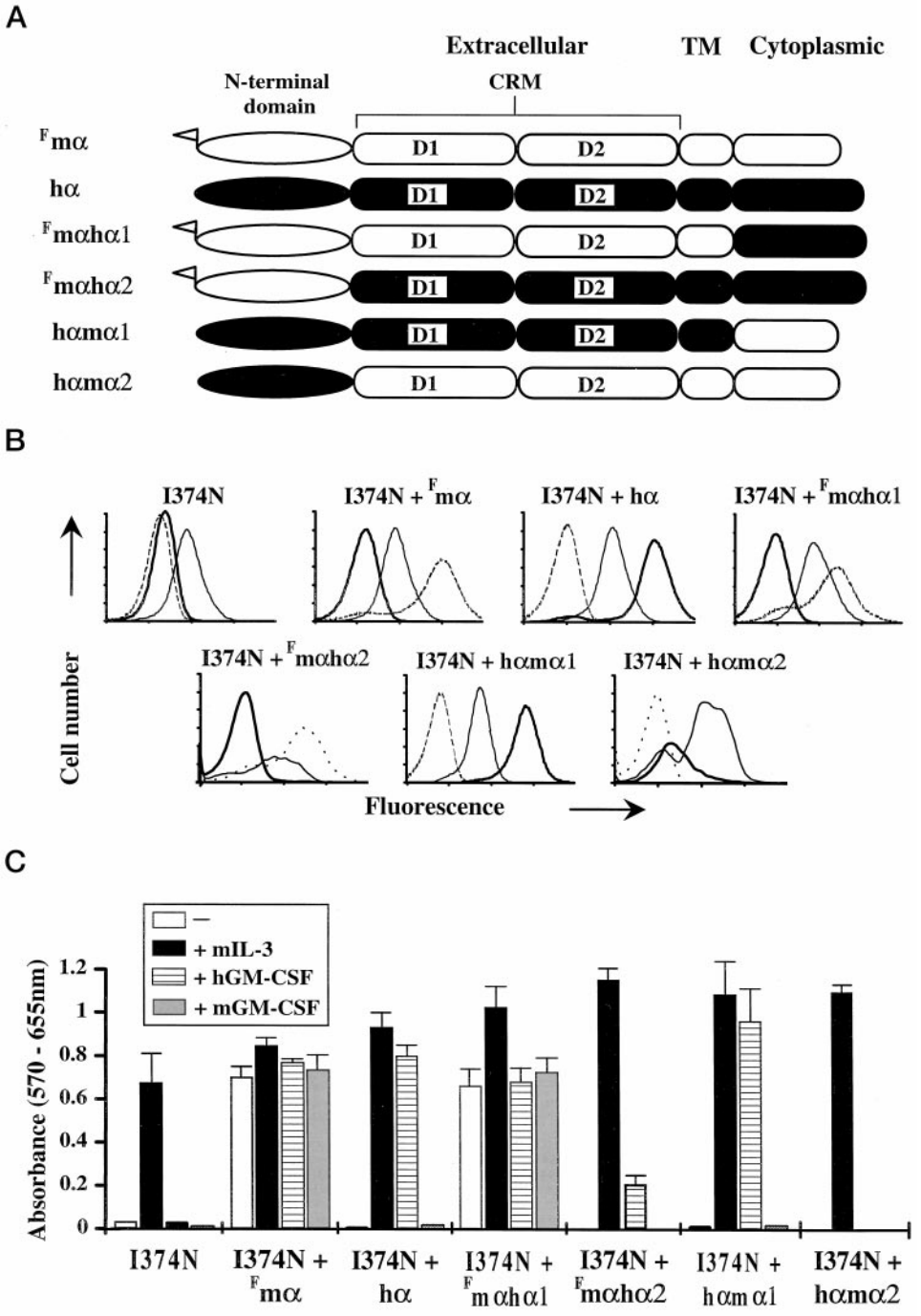

FIG. 7. **Analysis of BAF/I374N cells infected with retroviruses encoding chimeric mouse and human GMRα subunits.** *A*, schematic illustration of chimeric GMRα subunits. Regions from the mouse GMRα are shown in *white*, whereas regions from the human GMRα are shown in *black*. For comparison, the normal FmGMRα and hGMRα subunits are also shown. *B*, flow cytometric analysis of BAF/I374N cells that were superinfected with retroviruses encoding normal and chimeric GMRα subunits and stained with the anti-FLAG antibody M2 (*dotted line*), the anti-hGMRα antibody 8G6 (*thick solid line*), and the anti-hβc antibody 1C1 (*thin solid line*). *Axes* are as in Fig. 1*A*. *C*, proliferation assay of the BAF/I374N cells depicted in *B* in the presence of mIL-3 (300 units/ml), mGM-CSF (80 units/ml), or hGM-CSF (1 ng/ml) or in the absence of any factor, as indicated.

BAF-B03 or CTLL-2 cells (16), raising the possibility that cell type-specific signaling molecules are involved in its activation. In this study, we have employed retroviral expression cloning to identify the mGMRα subunit as one such molecule, since its introduction into BAF-B03 and CTL-EN (a derivative of CTLL-2) cells expressing the I374N mutant conferred factor independence. Importantly, the absence of the mouse GMR and IL-3R in CTL-EN cells indicates that the mechanism of activation of I374N does not require any subunits, apart from mGMRα, of these receptors. In contrast, another hβc mutant, V449E, that confers factor independence on both FDC-P1 and BAF-B03 cells (16) is not constitutively active when co-expressed with mGMRα in CTL-EN cells. This suggests that the I374N and V449E mutants are activated by fundamentally different mechanisms.

Physical Association of I374N and mGMRα—Co-immunoprecipitation experiments demonstrated that one effect of the I374N mutation in hβc is to induce constitutive association with mGMRα. The constitutive association between these subunits is reminiscent of a recent report in which hGMRα and

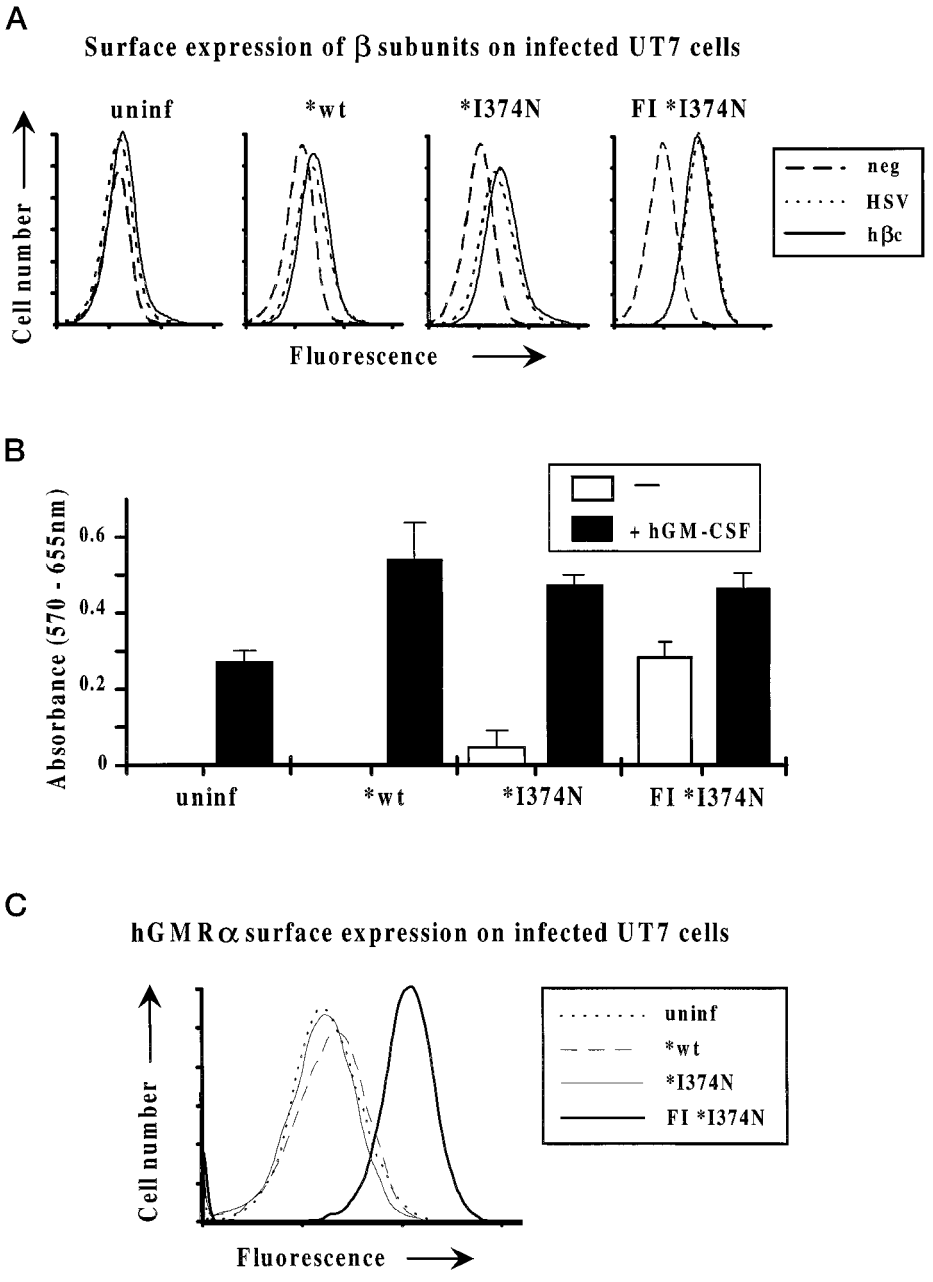

FIG. 8. **Analysis of human UT7 cells infected with the I374N mutant.** A, flow cytometric analysis of G418-resistant UT7 cells infected with retroviruses encoding the HSV-tagged (as shown by *asterisks*) wild-type and I374N β-subunits, except for the *panel* labeled *FI *I374N*, which shows staining of cells infected with the HSV-tagged I374N mutant and selected for factor-independent growth. Also shown are analyses of uninfected UT7 cells. Cells were stained with an irrelevant control antibody (*dashed line*), the anti-hβc antibody 1C1 (*thin solid line*), and the anti-HSV antibody (*dotted line*) by high sensitivity immunofluorescence. The *axes* are as in Fig. 1*A*. *B*, proliferation assay of the UT7 cells depicted in *A* in the presence and absence of human GM-CSF (2 ng/ml). *C*, flow cytometric analysis of hGMRα expression on the surface of the UT7 cells depicted in *A*. Cells were stained with the anti-hGMRα antibody 8G6 by high sensitivity immunofluorescence. The *axes* are as in Fig. 1*A*.

wild-type hβc were co-immunoprecipitated from cell lines in the absence of GM-CSF (32). Factor-independent association with hβc appears to be a unique property of GMRα, since similar preformed complexes could not be detected with hIL-3Rα or hIL-5Rα (32). This may in part explain the specific requirement for mGMRα, as opposed to mIL-3Rα, for constitutive activity of I374N.

We observed that deletions in the extracellular N-terminal domain of mGMRα abolished both the constitutive activity of I374N and the association between I374N and mGMRα, as well as mGM-CSF-induced proliferative signaling. While the corresponding domains of the hIL-3Rα and hIL-5Rα subunits have been reported to play a critical role in ligand binding (34–36), our demonstration that the N-terminal domain of mGMRα is required for association with the hβc mutant suggests that this domain may also play a role in receptor subunit assembly.

Our observation that the cytoplasmic domain of GMRα is needed for the activity of I374N was not unexpected, since deletion of the cytoplasmic domains of GMRα, IL-3Rα, and IL-5Rα renders these receptors inactive in proliferative signaling (33, 34, 37). Normally, however, α-subunit cytoplasmic truncations do not detectably affect the association of α- and β-subunits, since truncated α-subunits still form high affinity ligand-binding receptors (33, 34, 37), and a cytoplasmic truncation of hGMRα could still associate with hβc in the preformed hGMR complex described by Woodcock *et al.* (32). Thus, it is surprising that deletion of the C-terminal 14 amino acids of mGMRα also abolished the association between mGMRα and I374N. Nevertheless, this observation suggests that there may be a degree of interaction between the intracellular domains of α- and β-subunits and that the effect of such an interaction may only be detectable in the context of weaker extracellular interactions between mGMRα and I374N as compared with those between wild-type hβc and hGMRα.

Most importantly, however, the fact that (i) mGMRα associates with the I374N mutant but not with wild-type hβc and (ii) association of mGMRα mutants with I374N correlates with their ability to allow constitutive receptor activity suggests that induction of this association is essential for hβc activation. However, constitutive association of hGMRα with hβc *per se* is

not sufficient for receptor activation (32); thus, it is likely that the I374N mutation has additional effects such as mimicking a ligand-induced conformational change in hβc, as we have suggested previously (27, 38).

Determinants of the Species-specific Requirement for GMRα for the Constitutive Activity of I374N—In view of the ability of mouse GMRα to allow constitutive activity of I374N in mouse cells, it is somewhat surprising that co-expression of the human GMRα subunit with I374N in mouse BAF-B03 and CTLL-2 cells does not lead to factor-independent proliferation (Refs. 16 and 27; see also Fig. 7*C*). This is not due to the inability of I374N to interact with hGMRα because their co-expression in BAF-B03 and CTLL-2 cells results in the formation of a high affinity receptor and generation of a proliferative signal in response to human GM-CSF (16, 27). Moreover, I374N, like wild-type hβc (32), also efficiently co-immunoprecipitates with hGMRα in the absence of hGM-CSF (Fig. 4).

Our studies with mouse/human chimeric GMRα subunits showed that only the chimera containing the entire extracellular and transmembrane domains of mGMRα conferred factor independence on BAF/I374N cells, while in contrast, the human and mouse cytoplasmic domains were interchangeable. This suggests two possible explanations for species specificity: (i) that the extracellular domain of mGMRα interacts with I374N in a different manner from that of its human homologue, to allow formation of an active complex in the absence of ligand or (ii) that mGMRα interacts with a membrane-spanning accessory signaling molecule in a species-specific manner. The latter explanation would also suggest that species specificity might also be a function of the host cell species, *i.e.* that the accessory molecule might interact preferentially with the GMRα subunit of the same species. However, the fact that the I374N mutant was able to confer factor-independent proliferation on human GM-CSF/IL-3/erythropoietin-dependent UT7 cells argues against an exclusive requirement for murine GMRα. Moreover, the expression of hGMRα on the surface of the factor-independent cells was significantly up-regulated, suggesting that selection for factor independence also selected for hGMRα subunit expression. This is consistent with the notion that GMRα is involved in the constitutive activation of I374N in these cells also and that the species-specific requirement of GMRα for the constitutive activity of I374N may reflect the species of cell in which the mutant is expressed.

The relevance of the requirement for GMRα by I374N, and indeed other extracellular hβc mutants, to the activity of these mutants in primary hemopoietic cells should be noted. Retroviral infection of mouse fetal liver progenitors with the I374N mutant and another extracellular hβc mutant (FIΔ; Ref. 39) results in the formation of factor-independent cells of only granulocytic and monocytic lineages (40), the two major lineages controlled by GM-CSF (reviewed in Ref. 1). This observation is consistent with the notion that the constitutive activity of I374N and other extracellular hβc mutants is restricted to cells expressing GMRα.

Finally, the prediction that the extracellular (I374N) and transmembrane (V449E) constitutive mutants of hβc appear to act (at least in part) by inducing α-β or β-β dimerization, respectively, is interesting in light of evidence that the wild-type GMR/IL3R/IL5R may contain both α-β and β-β dimers (see the Introduction). It is tempting to speculate that each class of mutant might activate a subset of the multiple, overlapping signaling pathways activated by the wild-type receptor complex. Thus, they may constitute useful tools for dissecting receptor signaling.

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A Cell Type-specific Constitutive Point Mutant of the Common β-Subunit of the Human Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF), Interleukin (IL)-3, and IL-5 Receptors Requires the GM-CSF Receptor α-Subunit for Activation

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